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Determination of biogenic amines in fresh and processed meat by ion chromatography and integrated pulsed amperometric detection on Au electrode

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Abstract

A selective cation exchange chromatographic method, coupled to integrated pulsed amperometric detection, has been developed to quantify biogenic amines in fresh and processed meat. The method is based on gradient elution of aqueous methanesulfonic acid with post column addition of a strong base to obtain suitable conditions for amperometric detection. A potential wave-form able to keep long time performance of the Au electrode was set up. The analysis time is about 68 min. Amounts of tyramine, putrescine, cadaverine, histamine, agmatine, spermidine and spermine were measured, after extraction with perchloric acid. The method was used to determine analytes in fresh and processed meat. Analyte quantification was made with external calibration method after demonstration that matrix effects were not present. All analytes were identified in real samples except phenethylamine which is eluted in a zone of the chromatogram rich of interfering peaks. Repeatabilities, computed on their amounts in real samples, were better than 9% for all of them. Detection limits were computed according to the Hubaux–Vos method. The obtained values ranged between 0.70 and 2.12 mg/l corresponding to 7–21 mg/kg, low enough to determine all analytes in real matrices.

Keywords: Biogenic amines; Integrated pulsed amperometric detection; Au electrode; Meat products; Ion chromatography

1. Introduction

Biogenic amines (BAs) develop and accumulate in food and beverages as a consequence of enzymatic amino acids decarboxylation due to microbial enzymes and – to a lesser extent – to tissue activity (Halász, Baráth, Simon-Sarkadi, & Holzapfel, 1994; Shalaby, 1996). The determination of biogenic amines in fresh and processed food is getting of great interest not only for their potential risk for human health (Edwards & Sandine, 1981; Taylor, 1986) but also because they could have a role as chemical indicators of unwanted microbial contamination and processing conditions (Bover-Cid, Miguelez-Arrizado, Latorre-Moratalla, & Vidal-Carou, 2006; Ruiz-Capillas & Jimenéz-Colmenero, 2004). Main biogenic amines usually found in fresh and processed meat products are putrescine (PUT), cadaverine (CAD), histamine (HI) and tyramine (TY) (Demeyer et al., 2000), while natural polyamines (PAs) spermidine (SPD), and spermine (SPM) content slightly changes during storage or processing (Hagen, Bauer, & Paulsen, 2005; Hernández-Jover, Izquierdo-Pulido, Veciana-Nogués, Mariné-Font, & Vidal-Carou, 1997). Several authors highlighted the relationship between hygienic quality of raw material and aliphatic amines accumulation: changes in concentration of PUT and CAD are known to correlate with the microbial spoilage, storage temperature and storage time of meat (Edwards, Dainty, & Hibbard, 1983; Dainty, Edwards,

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Hibbard, & Ramantanis, 1986; Durlu-Özkaya, Ayhan, & Vural, 2001), while TY is usually the most common amine found in fermented sausage and dry cured meat products because it is mainly related to the activity of fermentative lactic acid bacteria (Alfaia et al., 2004; Bover-Cid, Izquierdo-Pulido, & Vidal-Carou, 2001; Rokka, Eerola, Smolander, Alakomi, & Ahvenainen, 2004).

For all these reasons biogenic amines profile could be an important index in quality assurance of fresh and processed meat (Bauer, 2006); however the complexity of food matrix, the presence of potential interferences and the occurrence of several biogenic amines simultaneously are typical problems encountered in the analysis of food for biogenic amines.

The analytical methods for determining BAs and PAs are usually based on GC and HPLC separation coupled to different detection techniques such as FID (Hwang, Wang, & Choong, 2003), fluorimetry (Vidal-Carou, Lahoz-Portoles, Bover-Cid, & Marine-Font, 2003; Kirschbaum, Busch, & Bruckner, 1997; Tamim, Bennett, Shellem, & Doerr, 2002; Salazar, Smith, & Harris, 2000; Saito, Horie, Nose, Nakagomi, & Nakazawa, 1992), conductometry (Cinquina et al., 2004; Draisci, Cavalli, Lucentini, & Stacchini, 1993), mass spectrometry (Saccani, Tanzi, Pastore, Cavalli, & Ray, 2005). Pulsed amperometry (Hoekstra & Johnson, 1999; Pineda, Knapp, Hoekstra, & Johnson, 2001; Sun, Yang, & Wang, 2003), also with dedicated wave-form (Pastore et al., 2005), is used. The main drawbacks of the majority of these methods are related to pre-column, post-column or on-column derivatisation process leading to an overall long analysis time, and low reproducibility owing to the stability of the derivatisation products. Pre-derivatization comply a series of manual steps time consuming and that introduce many imprecision contributions. On the other hand it may add certain selectivity. Post-column derivatization has the advantage that is in line, but add complexity to the instrumentation, and system must be set up in order to reduce the contributions to band widening. Changing of pH with a post-column system is simple, easy and quick.

Conductometric detection does not involve derivatisation step but uses a chemical suppression of the eluent conductivity leading also to loss of some analytes and detect also alkaline and alkaline-earth cations usually present in real matrices. Amperometric detection is less affected by these drawbacks although electrode poisoning effects may arise.

More recently proposed methods for BAs analysis are the traditional derivatization with dansyl chloride in cheese (Innocente, Biasutti, Padovese, & Moret, 2007) which uses an extraction, derivatization and a second extraction to eliminate the interferents, that is a very long preparation step. The authors do not cite LOD and dynamic range. Capillary electrophoresis with preceding evaporation was also used, obtaining good limits of detection (0.2–0.6 mg/l as 3 s) and repeatability (Ruiz-Jiménez & Luque de Castro, 2006). Micellar electrokinetic chromatography with electrochemical detection is another approach (Bose et al., 2004) in serum samples. The LODs appear extremely low, compared to chromatograms showed at a concentration 1000 times higher. Very few papers are dealing with meat samples, being a very complex matrix.

This work deals with the developing of a chromatographic method with amperometric detection for monitoring the complete profile of BAs and natural PAs in fresh and processed meat. This approach was successfully applied to the analysis of biogenic amines containing aromatic groups in cocoa samples (Pastore et al., 2005) and ensures good sensitivity. In this context, the potential wave-form was adapted to the meat matrix and separation conditions were drastically modified. The method was validated and its application to biogenic amines in fresh and processed meat was demonstrated.

2. Experimental

2.1. Chemicals

All reagents were of analytical grade and were used as purchased: TY >99% (Fluka, Milan, Italy), HI base >97% (Fluka, Milan, Italy), 2-phenylethylamine >99% (Fluka, Milan, Italy), PUT dihydrochloride >99.0% (Sigma-Aldrich, Milan, Italy), CAD dihydrochloride >98% (Sigma-Aldrich, Milan, Italy), agmatine (AGM) sulfate salt 99.0% (Fluka, Milan, Italy), SPM tetrahydrochloride >99.5% (Fluka, Milan, Italy), SPD trihydrochloride >99% (Sigma–Aldrich, Milan, Italy), $HClO_4$ 70% RP Normapur (Prolabo, France), NaOH (Riedel de Haën-Fluka, Milan, Italy), methanesulfonic acid 99% (Sigma–Aldrich, Milan, Italy). The solutions were prepared with milli Q water (Elix5, Milli Q Gradient System, Millipore, Milan, Italy, resistivity 18.2 MΩ · cm). Standard solutions of BAs and PAs were prepared in $HClO_4$ 0.1 M at 1000 mg/l and stored at 5 °C. For the evaluation of recovery from processed meat samples, a more concentrated solution was used (containing about 200 mg/l of SPD, 1 g/l of SPM, 2 g/l HI, 3 g/l of CAD, 7 g/l of PUT, 9 g/l of TY), to avoid the addition of a large amount of solution.

2.2. Ion chromatography

A Dionex (Sunnyvale CA, USA) GP50 gradient pump equipped with an EG40 electrochemical detector and a LC25 oven was used as chromatographic system. All devices were controlled by the Chromeleon software, 6.50 version. The column used was a Dionex IonPac CS17 (2 × 250 mm, 5 μm) with a pre-column IonPac CG17 (2 × 50 mm, 5 μm) thermostated at 40 °C. The eluent was methanesulfonic acid (MSA) 1.6 mM for 14 min, linearly increased to 6 mM in 34 min and then linearly increased to 40 mM in 4 min. This last concentration was maintained constant for 6 min. The chromatographic run was preceded by 15 min of equilibration at 1.6 mM MSA. The flow rate was 0.40 ml/min. A post column addition of 0.1 M NaOH

was made by using a Bio-Rad (Richmond, USA) 1350 T pump at 0.40 ml/min. NaOH was directly mixed to the eluent with a "T" junction followed by a single bead string reactor, 30 cm × 0.5 mm i.d., and a knitted coil, 30 cm × 0.5 mm i.d., obtaining an operative measured pH close to 12.7. The injected volume was 10 μl. The flow-through electrochemical cell (1.5 μl, Sunnyvale CA, USA) consisted of a 1.0 mm diameter Au working electrode, a titanium counter electrode and a pH-Ag/AgCl combination reference electrode. The adopted wave-form and the corresponding integration interval is reported in Table 1.

2.3. Procedures

2.3.1. Extraction of amines from samples

Samples of dry-cured ham, fermented sausages and dry cured bellies were suitably cut up and homogenized. Fresh pork meat (from *longissimus dorsi* muscle) selected from the inner part of the muscle, was used as "blank" sample for PAs natural occurring amount and to confirm the presence of potential interfering compounds. Amines were extracted in ultrasonic bath (Branson mod. 2200) for 10 min with 20 ml of 0.1 M $HClO_4$ and centrifuged at 4500 rpm for 40 min (Serafini mod. 322, Milan, Italy) to separate the fats. Possible differences between extracted analytes and interfering species were checked starting from 1, 2 and 5 g of processed meat samples. The highest ratio analytes/interferents was obtained with 2 g and consequently this amount was used in all successive experiments. The aqueous solution was then filtered with a 0.45 μm regenerated cellulose filter (RC, Alltech, Milan, Italy) prior injection. A second extraction with 5 ml of $HClO_4$ was made to verify the completion of extraction.

2.3.2. External calibration

External calibrations were performed using solutions containing all analytes, prepared from concentrated solutions of single amines (1000 mg/l, in $HClO_4$ 0.1 M) in the range 0.5–20.0 mg/l.

Table 1
Detection wave-form

IPAD detection sequence		
Time (s)	Potential[a]	Integration
0.00	−0.25	
0.20	−0.25	Begin
0.35	0.55	
0.45	0.55	
0.60	−0.25	
0.80	−0.25	End
0.82	−1.00	
0.84	−0.25	
0.86	−1.00	
0.88	−0.25	
0.90	−1.00	
0.92	−0.25	
0.94	−1.00	
0.96	−0.25	
0.98	−1.00	
1.00	−0.25	

[a] (V vs. Ag/AgCl).

2.3.3. Standard additions calibration

Three standard additions of the analytes were also made, in the extract, to verify the absence of matrix effect. Additions were chosen to obtain concentrations very close to the estimated amounts in the sample. Standard additions were: TY, 0.30, 0.60, 0.90 mg/l; PUT, 1.00, 2.00, 3.00 mg/l; CAD, 0.80, 1.60, 2.40 mg/l; HI, 0.30, 0.60, 0.90 mg/l; SPD, 0.30, 0.60, 0.90 mg/l; SPM, 0.50, 1.00, 1.50 mg/l. AGM was not added in real samples because absent.

Recovery tests. Extraction yield was evaluated by adding 50 μl of a standard solution containing TY (9.5 g/l), PUT (7.0 g/l), CAD (3.2 g/l), HI (1.7 g/l), SPD (200 mg/l) and SPM (1.0 g/l) to 2 g of processed meat. This procedure was repeated twice. Samples were homogenized before extraction made as previously described.

3. Results and discussion

3.1. Choice of the potential wave-form

A potential wave-form for the determination of biogenic amines in chocolate was developed in a previous paper (Pastore et al., 2005). It must be underlined that the amines in chocolate are prevalently aromatic (dopamine, serotonin, etc.) whilst those found in meat are prevalently alkyl-amines, so that a difference in response is reasonable. Consequently, the wave-form was modified to optimize repeatability and linear response of the considered amines. In particular, both detection and cleaning potential steps were increased (see Table 1 for the used wave-form).

3.2. Chromatographic separation

Chromatographic separation is affected by column temperature. The chosen temperature was 40 °C (the highest possible to keep the column safety unchanged) as it produced sharper peaks and improved efficiency and selectivity.

Fresh pork meat and processed meat were analysed. Matrices were quite similar, whilst biogenic amines content varies by a great extent in processed meat, depending on manufacturing process and microbial factors: for this reason processed meat coming from different manufacturing technologies such as fermented dry sausages and dry cured ham were chosen. Sample matrix is quite complex so that a very slow gradient was required to separate amines from interfering peaks. Fig. 1 shows two chromatograms relative to a standard solution (a) and to a processed meat sample (b). Baseline drift is a consequence of the gradient. The two steps at 40–45 min and 61–66 min were decreased when eluent and post-column solutions were prepared with freshly obtained ultra-pure water. Baseline drift is therefore likely due to oxidation of organic molecules present in water eluted at a given eluent composition. The comparison of the two chromatograms shows that, as often occurs, matrix

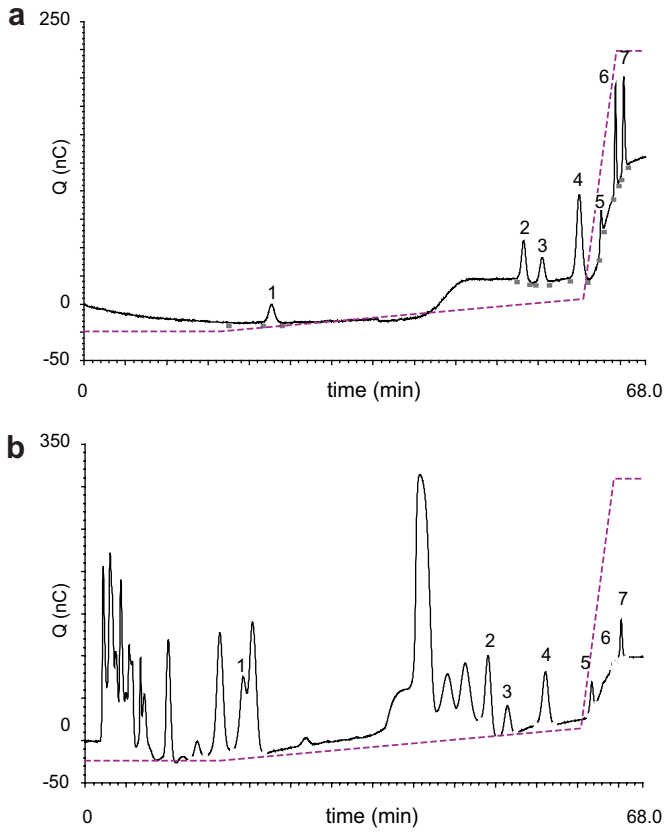

Fig. 1. (a) Chromatograms of a standard solution of the 7 amines and (b) of a processed meat sample. (1) TY 1 mg/l, (2) PUT 1 mg/l, (3) CAD 0.5 mg/l, (4) HI 0.5 mg/l, (5) AGM 0.5 mg/l, (6) SPD 0.5 mg/l and (7) SPM 0.5 mg/l. Experimental conditions: column Dionex CS17 with CG17 precolumn; methane sulfonic acid gradient (see procedure section); flow rate 0.40 ml/min; post-column NaOH 0.1 M at 0.40 ml/min; injected volume 10 μl; T, 40 °C. IPAD conditions as in Table 1. Dashed line represents the applied eluent gradient.

composition influences retention times of the analytes. In this case, the effect is evident for all peaks except SPD and SPM, too strongly retained to be conditioned by the increased eluting strength given by the matrix components. TY is not completely resolved, despite the chosen gradient.

3.3. Calibration

Calibration was performed in the range 0.5–10.0 mg/l for CAD, HIS, AGM, SPD and SPM and in the range 1.0–20.0 mg/l for TY and PUT. For calibration every concentration level was repeated in triplicate. The correlation is linear for most amines, in the explored range. HIS, SPD and SPM were better fitted with a second order regression model. Calibration results are reported in Table 2. A non linear response was already previously found for the amperometric detection of some biogenic amines (Hoekstra & Johnson, 1998). This fact may be justified with the mechanism of oxidation, involving the amines adsorption on Au electrode surface. Calibration with standard addition method was also performed to investigate the possibility of a matrix effect (AGM was not added because absent in the real matrix). Standard additions were made on the extract at three concentration levels, close to the estimated amounts present in the sample, to minimize the imprecision of extrapolated data. Calibration parameters are reported in Table 2. A linear regression was satisfactory for all the analytes owing to the limited concentration range. The comparison of the two kinds of calibration evidences the statistical identity of the slopes of the two calibration modes. In the case of second order regressions (HIS, SPD and SPM) the range of concentrations explored with standard addition method coincides with the linear range of the external calibrations. This fact ensures the absence of matrix effect on the signal and enables the use of an external calibration, applicable for all the meat samples of this work, in the explored concentration range. The external calibration procedure was obviously preferred for the lower analysis time needed.

Table 2
Figures of merit of the method for external and internal calibration

	TY	PUT	CAD	HIS	AGM	SPD	SPM
External calibrations[a]							
b_0	0.05	6.9	−0.38	1.4	0.34	−0.23	−1.7
b_1	9.8	16.18	13.64	39.1	6.55	19.6	21.8
b_2	–	–	–	−0.56	–	−0.75	−0.79
$s(b_0)$	2.1	7.0	1.3	5.7	1.0	4.2	5.1
$s(b_1)$	0.2	0.68	0.25	3.3	0.25	2.4	2.9
$s(b_2)$	–	–	–	0.30	–	0.22	0.26
$s_{y/x}$	5.4	12	3.3	10	2.8	7.2	8.8
R	0.997	0.989	0.998	0.997	0.994	0.987	0.98
Calibratios with standard addition method[b]							
b_0	77.1	70.7	29.23	59.4		0.75	13.24
b_1	13.0	18.7	13.36	42.6		18.17	20.3
$s(b_0)$	5.7	2.2	0.66	4.3		0.23	0.96
$s(b_1)$	2.0	1.2	0.44	7.7		0.42	1.0
$s_{y/x}$	12	4.5	1.4	8.9		0.48	2.0
R	0.89	0.98	0.994	0.87		0.997	0.987
Critical value (x_c) and limit of detection (x_d) in mg/l for a confidence level of the 95%[c]							
External calibration							
x_c	0.74	1.23	0.35	0.40	0.50	0.37	0.38
x_d	2.12	1.58	0.75	0.95	1.10	0.70	0.74
Standard additions							
x_c	1.84	0.48	0.21	0.42	–	0.05	0.20
x_d	3.60	0.94	0.40	0.85	–	0.11	0.39
Recovery[d]							
Addition (mg/kg)	240	170	80	42	40	3	25
Recovery (%)	90	103	92	74	66	61	92
RSD (%)	3.4	2.0	1.8	5.4	6.6	4.9	2.7

[a] Regression parameters for five concentration levels with three repetitions ($n = 15$). b_0, b_1, b_2, coefficients respectively of order 0, 1, 2; $s(b_0)$, $s(b_1)$, $s(b_2)$, standard deviation of the regression coefficients; $s_{y/x}$, standard deviation of residuals; R, correlation coefficient.

[b] Regression parameters of the standard addition method. The extract was added at three concentration levels (three repetitions each).

[c] Critical value (x_c) and limit of detection (x_d) in mg/l for a confidence level of the 95%.

[d] Recovery obtained for addition of the analytes to the sample before extraction. The value are mediated on two independent samples, repeated twice ($n = 4$).

3.4. Analysis of real matrices

Various samples were analysed with the proposed method. In Fig. 2 an example of dry-cured ham is reported, and Table 3 reports the found analyte amounts for the various samples. Each value is the mean of three samples coming from different aliquots of the original homogenized sample. The standard deviation of the mean varies between 1.2% (TY) and 11% (PUT).

TY, PUT and CAD were the most important amines found in long aged meat samples, ranging from 38 to 295 mg/kg for TY, from not detected to 331 mg/kg for PUT and from not detected to 81 mg/kg for CAD, although in variable amounts depending on the samples. The variability of amine contents in meat samples, in particular in fermented and dry-cured sausages, could be explained on the basis of the technological process, raw material hygienic quality and contaminations accidentally occurring during the process (Suzzi & Gardini, 2003; Virgili, Saccani, Gabba, Tanzi, & Soresi Bordini, 2007). A long ageing time can also affect the final content of biogenic amines – particularly TY – and justify the large range observed in dry cured meat samples, purchased at the end of ripening time in retail markets. Biogenic amine content in fresh meat samples was generally much lower than in long aged meat samples as their microbial contamination is generally more controlled. PUT, CAD, HI as well as AGM were not detectable, however a noticeable level of TY was observed. As expected small amounts of SPD and SPM were found in all the meat samples both fresh and processed (Kalac, 2006).

Data resulting from the monitoring was in agreement with others and show that the BA and PA content depends on the manufacturing process and micro-flora growth (Virgili et al., 2007; Saccani et al., 2005; Alfaia et al., 2004; Demeyer et al., 2000).

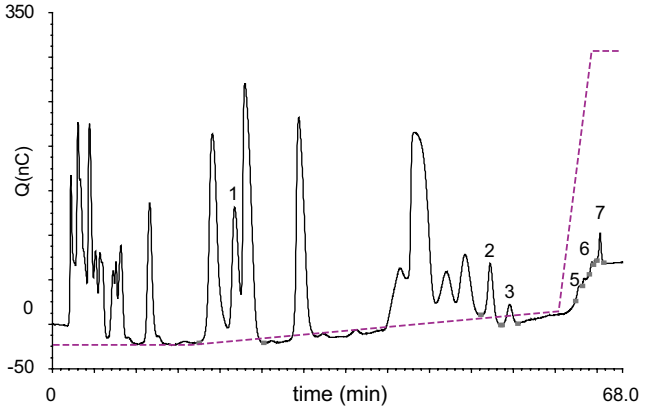

Fig. 2. Chromatogram of dry cured ham. Experimental conditions as in Fig. 1. Dashed line represents the applied eluent gradient.

3.5. Recovery

The recovery of analytes was first checked by making a second extraction on the same sample. No peak of the analytes was found so that we assumed the extraction procedure was quantitative. The recovery was then evaluated by adding a known amount of standard amines to the meat sample before extraction. Additions were made adding an amount approximately equal to that estimated with the calibration curve in the real sample. Table 2 gives the recovery values. Recoveries were quite good for TY, PUT, CAD and SPM. Lower yields were obtained for HI, AGM and SPD, because found amount of these analytes was close to the detection limit and addition to the matrix should be in the same concentration range. Their recoveries were much better at higher concentrations.

3.6. Limits of detection

As already stated, the quantitative determination of the studied BAs and PAs was made with an external calibration. The determination of the limit of detection (x_d) was therefore obtained on the basis of the statistics of a calibration plot at low concentration values (Hubaux & Vos, 1970). Table 2 reports the critical concentration level, x_c, and the x_d for all amines, obtained in the described conditions. The reported values are sufficiently low to accurately quantify BAs and PAs concentration usually present in

Table 3
Amine amounts detected and relative standard deviation (RSD %) in various processed meat and one fresh meat samples

		TY	PUT	CAD	HIS	AGM	SPD	SPM
Dry fermented sausages	amount (mg/kg)	273	144	81	57	19	2.0	18
	RSD (%)	8.6	5.2	2.6	10	8.0	4.8	6.9
Sopressa (typical italian fermented sausage)	amount (mg/kg)	214	231	66	–	–	2.0	17
	RSD (%)	7.9	6.2	2.3	–	–	4.2	7.2
Dry-cured ham	amount (mg/kg)	104	97	64	–	14	4.7	16
	RSD (%)	1.2	4.8	1.9	–	3.1	3.3	4.4
Dry-cured belly	amount (mg/kg)	295	331	6	114	–	1.3	16
	RSD (%)	9.3	6.5	10	6.0	–	11	6.0
Fresh meat	amount (mg/kg)	38	–	–	–	–	1.4	19
	RSD (%)	2.5	–	–	–	–	10	6.7

Means of three independent samples.

meat. Table 2 reports also x_c and x_d obtained with the standard addition method as some authors proposed a matrix-considering in-house validation concept for analytical methods which takes into account the uncertainty due to matrix- and time-induced deviations (Julicher, Gowik, & Uhlig, 1998). They proposed to apply the same approach of the external calibration to the unknown matrix so that standard BAs and PAs are added at concentrations just above the level of precision, so that two contiguous signals might be detected as statistically different. In our case the x_c and x_d values found by standard additions are similar to those obtained with the external calibration. The observed differences may be ascribed to a different concentration range considered in the two calibration modes. With the exception of TY the results obtained with the standard addition method are slightly better probably for a better signal repeatability at the considered concentration range. On the other hand, the analysis time becomes too long and this justifies our choice to use the external calibration renouncing to the lower x_d.

Limits of detection obtained by other authors were quite similar, with the exception of Saccani et al. (Saccani et al., 2005) in the case of mass spectrometric detection and of Bose et al. (Bose et al., 2004) in serum samples. On the other hand all of them were calculated as three times the standard deviation of the blank which gives very lower values than those calculated from the calibration curve, and are not operative values.

4. Conclusions

The use of a reductive multiple step potential wave-form after the current integration step applied to Au electrodes, together with a gradient separation by cation exchange, allows a selective and sensitive determination of seven BAs and PAs usually present in meat samples. The sample preparation is very simple consisting in extraction of the analytes with perchloric acid. The quantification of the analytes in real samples by external calibration was used after demonstration that matrix effects were not present. Detection limits and repeatabilities of the measurements are good enough to give robust results and to propose the analytical approach for the determination of the studied BAs in meat samples.

The most important improvement of this approach is the possibility of injecting directly the perchloric acid extract without any further treatment. This approach can be therefore regarded as a direct determination of BAs and PA in a complex matrix as meat. Moreover, the chosen gradient, shows a better resolution that evidences the presence of unknown peaks whose identification will be the scope of a future work.

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